Experimental and mathematical exploration of the dynamic behavior of simple gene networks

Ph.D. Thesis

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CONTENT

PREFACE

The majority of natural phenomena can be explained without the invocation of an involved mathematical language. Notwithstanding, a mathematical formalism is often required to fully comprehend simple, well-defined or homogenous objects or events (atoms, hydrodynamic self-organization). While such formalism has permeated physical sciences increasingly, life sciences evaded this tendency largely, concerning in vivo processes. The recent reappearance of a *combinatorial* mathematical-experimental research in cellular biology is spurred by the developments in molecular biological methodology in the last decade and partially by the advance in qualitative dynamical and probabilistic mathematics and computational facilities. Despite this facilitation, problems such as, incomplete description of cellular components and interactions, considerable variability and noise in biological processes have to be tackled with. To overcome these difficulties and to make the system amenable to mathematical assistance the following guidelines were followed in the design of experimental systems and in the choice of theoretical approaches:

1. The biological system to be studied has to be chosen rationally or by exploratory experimentation so that the combinatorial approach can be applied to its best advantage. From a heuristic reasoning the system has to be simple and well-defined yet we have to presume that it will display properties which can be captured only or optimally by a mathematical model.
2. The exploration of the selected system should shed light on general principles of in vivo processes or should help to understand the functioning of specific but relevant cellular networks.
3. The mathematical formulation of the biological system has to be the best possible approximation of the underlying processes. I have assumed that the biological processes I studied can be translated into the terms of chemical reaction kinetics governed by the law of mass action. To account for the considerable variability and for the effects of inherent fluctuation in living organisms, the deterministic mass action models were extended with stochastic terms. Actually, the main goal of my research was to study the effects of fluctuations on the behavior genetic feedback systems.

Based on the above guidelines simple gene circuits of transcription factors were designed in *E. coli* and *S. cerevisiae.* Simple circuits incorporating negative or positive feedback are conceived as elementary and ubiquitous modules of larger gene networks, which orchestrate basic cellular processes such as cell cycle, biorhythms and development. Taking into consideration the known mechanisms of gene expression in prokaryotes and eukaryotes, a tetracycline-modulated transcriptional repressor or activator was used to construct negative and positive feedback circuits, respectively. The exploration of these systems with

appropriate reporter genes, revealed that expression levels of proteins in cells can be described only with a probability distribution. The shape of the distribution is affected by fluctuations. Negative feedback in genetic circuits can reduce the variance of the distribution, while positive feedback generates a switch, where the transition between two stable states is induced by noise. Both of these properties can be captured only by mathematical models, which account for fluctuations

The introduction starts with the description of approaches that aim at reducing the gap between theoretical modeling and experimental analysis of gene networks. The second part summarizes the basic features of genetic and cellular networks in different organisms, where a special attention is paid to the structural (topological) and dynamical features of gene and cellular networks. Fluctuations are essential in shaping the dynamical features, however, fluctuations can be described mathematically by a variety of ways and it is often difficult to find correspondence with the physical-chemical terminology of non-ideal (bio)chemical reactions. The third part is an overview of mathematical formalisms which can be or have been used to model gene networks and take fluctuations into consideration. A short mention is made about the basic properties of gene expression which affect the design of gene networks and interpretation of their behavior.

The summary of results and the discussion accounts for the recent developments in the understanding of genetic switches and fluctuations in gene networks.

It is important to take into account that components of cellular networks have often a distinct spatial distribution. Biochemical reactions are often compartmentalized or heterogeneously distributed in cells. Nuclear transport is probably the most well-characterized process, which influences the spatial distribution of cellular network components. My current research focuses on how nuclear transport affects the dynamics of cellular networks.

INTRODUCTION

How to lessen the gap between mathematical models and molecular biological experiments? The combinatorial approach

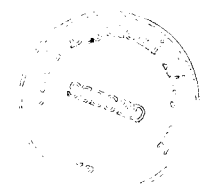
Neither mathematics was invented to shed light on the principles of life, nor the usual molecular biological experiments were carried out with the purpose to provide data for abstract mathematical models.

The advantage of combining mathematical and experimental approaches in analysis of biological processes is connected with the insufficiency of mathematical formulation and experimental characterization of biological processes. The applied mathematical language is not always consistent with *in vivo* reaction kinetics and the experimental description of biological processes is often not adequate to be translated into equations. On the other hand, experiments that address the dynamics of network of interacting proteins, often have to be designed by applying principles borrowed from physical chemistry and dynamical systems theory. The *combinatorial* approach allows to design experiments and analyze data, which in turn help to model adequately and to comprehend the dynamics of biological processes.

Mathematica ancilla biologiae

In the earlier stages of a given field of science, its internal logic is efficient in solving its own problems. For example, setting up the periodic system of elements required mostly a good combinatorial logic. At a later stage, even simple natural phenomena are explained by a rather involved mathematical language. A traveling solitary wave in a river can be understood by a highly nonlinear partial differential equation (Debnath, 1997). The Benard-instability is understood by the combination of deterministic and advanced stochastic approaches. Similar relation is found in the explaining the color difference of silver and gold by the Dirac's equation, and oscillatory chemical reactions by dynamical systems theory. These models explain accurately measured experimental data

A common feature of these models is that they are strongly built on accurately measured experimental data. Once these models are established, their generalization can ensue on a pure theoretical basis. However, molecular biology is a rather young science with few accurate and generally valid data concerning in vivo processes. A realistic perspective of the applicability of mathematics in this field is given by Blumenfeld. "Difficulties of making adequate physical description of the basic characteristics of macromolecular and subcellular structures of living matter are connected not only with their kinetic out-of-equilibrium state, but also with the fact that this state changes during their functioning and due to rather subtle



changes of parameters as temperature or pH. This often makes it meaningless to utilize many ideas and equations, which have been successfully applied in the studies of simpler molecules. The development of *quantitative and semi-empirical theories*, capable of playing the role which Vant' Hoff's approach plays role in thermodynamics and Arrhenius's approach in kinetics, represents one of the most important tasks of the science of biological physics. For many biological phenomena it is as yet impossible to find not only the solution but even the formulation of the corresponding physical problems." (Blumenfeld, 1981).

Whereas some scientists are of the opinion that the present laws and concepts of quantum mechanics will have to undergo modifications before they can be applied to the problem of life (Wigner, 1962). Others, think that the known principal laws of physics are quite sufficient for a complete description and understanding of the structure and functioning of all existing biological systems. The foundation of laws of chemistry does not, in principle, require any new postulates. The introduction of new postulates seems to be always connected with new world constants. When we pass from the laws of microphysics to those of microphysics, we have to introduce the postulates of quantum mechanics and a new world constant, h. The light velocity was only raised to the rank of a fundamental natural constant by relativity theory. "New physics" means the abandonment of the general validity of previously accepted fundamental principles required by experimental facts which, although obtained under clear and defined conditions, are in disagreement with the conclusions of the theory (Eigen, 1971).

Although molecular biology underwent a rapid development in the last two decades the main idea problems remain with the treatment of in vivo systems with theoretical tools. Whether such new theories would require invention of new principles is not known. However, it seems quite likely that new theoretical tools have to be developed to tackle the complexity of even simple biological processes.

Design possibilities for the combinatorial approach

A major difficulty in the application of mathematics for biological processes stems from the lack of well-characterized cellular networks: parameters are missing and many components remain unidentified. Recently the genomic and proteomic methods provided a wealth of physical interaction data. However, their accuracy is not sufficient for identification of single-cell dynamical interactions owing to the possibility of large cell-to-cell variation of biological process (e.g. switch due to a positive feedback). In addition, many processes are invisible to these methods such as the nucleo-cytoplasmic transport and spatial distribution of kinase - phosphatase reactions (Ohno, 2000). The following approaches have been used to circumvent the above difficulties.

1. In vitro reconstruction of a reaction network from the components of the cellular network. A biological process can be reconstructed in vitro from a minimal number of necessary compounds. For example, actin based motility as induced by Listeria requires five purified proteins (Loisel, 1999). A combinatorial approach was adopted e.g. to study how dynamic patterns are formed from microtubules by the action of modified molecular motors (Nedelec et al, Surrey et el). Oligomeric motor complexes were made by mixing biotinylated kinesin and streptavidin or GST-Ncd and anti-GST monoclonal antibodies. At high Ncd (minus-end-motor) concentrations asters are formed while kinesin formed vortices at intermediate and asters at higher concentration. When both motors are added the observed pattern depends on the ratio of the two motors.

2. In vivo reconstruction of the network topology using synthetic-compounds. Such approach is also termed as forward engineering because synthetic gene or cellular network preserve the original topologies of a network to be studied, however, the components are synthetic in order to attain more complete control of system parameters. Precise perturbations can be introduced to the system without interference to, or from, ancillary processes in the cell. Thus, synthetic gene networks allow the accurate comparison of theoretical and experimental results, and can, in theory quickly reveal possible principles and motifs of cellular regulatory properties (Gardner, 2000). For example, a synthetic genetic switch and an irregularly oscillating gene network was designed in *E. coli.* (Gardner, 2000; Elowitz, 2000).

3. Study of in vivo network. To extract information from a cellular network it can be modified or perturbed. Reducing or increasing the concentration or activities of various components provides a milder way of perturbing a network. Replacement of wild-type compounds by mutants is of interest when the mutations affect specific activities of compounds. This approach is well-suited for networks, which are characterized in detail. Such a network governs the chemotaxis of bacteria. The analysis of robustness of this system is in qualitative agreement with the experimental data (Alon, 1999). These results also suggest that a major goal of this field is to provide good qualitative predictions for a specific network which is even more valid for slightly less characterized networks.

Various properties of biological processes can be predicted based on network topology and parameters, and the predictions can be tested experimentally. However, the description of network topology is frequently insufficient to allow such testing. In these situations an alternative approach can be the applied to understand the dynamics of the given network. Possible physical-chemical models for a cellular network to be characterized are developed to

a point where they can be distinguished and predictions are derived from the corresponding mathematical models. Subsequently, experimental data are obtained and used to decide which model fits the cellular process properly. I adopted this approach to study the dynamics of nucleocytoplasmic transport. An important characteristic of the study of networks *in vivo,* is that new techniques generally have to be developed to measure intracellular activities. In this case, I developed a gel-shift assay for measuring unidirectional Brownian motion across the nuclear pore.

Properties of cellular networks and feedback control

Cellular and gene networks

Definition. Genes and their products known to play a role in the biological process to be modeled define the set of *network components*. The interactions among the products result in changes of amount, activity and localization of the gene products. For example, a kinase can decrease the activity or amount of an enzyme, while a transcriptional activator increases the amount of mRNA. These negative and positive interactions build up the *interaction map* or *topology* of the network. When the network components show spatially inhomogeneous diffusion and reaction properties the appropriate compartmentalization has to be superposed to the topology. An example for a cellular network can be seen on Figure 1. The distinction between cellular and gentic network is arbitrary. generally, gene network is used when transcriptional factors can be found among network components.

Network topology. Analysis of the network topology in various organisms shows that certain regularities can be found for the network topology. An average of 5-6 interactions per protein are found in two-hybrid screens of *C. elegans* (Tucker, 2001; Walhout, 2000). 72% of all interactions are between two partners of the same functional class such as cell-cycle regulation or protein translocation etc. When the interactions are randomized only 12% of all interactions belong to the same class. Proteins in certain functional groups, such as cell-cycle regulation, transcription and chromatin regulation interact with proteins of many other classes, consistent with their central roles in the cell (Schwikowski, 2000). Analysis of the distribution of the interactions among different components can define a specific class of large-scale network topology. For example, the representation of a metabolic network of is built up nodes, the substrates, that are connected to one another through links, which are the actual metabolic reactions. A large-scale topology can be described by an inherently random and uniform exponential model or by a highly heterogeneous scale-free model. The random model introduced by Erdös and Rényi assumes that each pair of nodes (that is, components)

in the network is connected randomly with probability p, leading to a statistically homogeneous network, in which, despite the fundamental randomness of the model, most nodes have the same average number of links, $<k>$. In particular, the connectivity follows a Poisson distribution that peaks strongly at $<k>$ implying that the probability of finding a highly connected node decays exponentially ($P(k) \approx e^{-k}$ for $k>><k>$). On the other hand for a scale-free network $P(k)$ follows a power law, $P(k) \approx k^{-\gamma}$. Unlike exponential networks, scale-free networks are extremely heterogeneous, their topology being dominated by a few highly connected nodes (hubs) which link the rest of less connected nodes to the system. A comparative analysis of the metabolic networks of 43 organisms shows that, they belong to the class of scale-free network despite significant variation in their individual constituents. For instance, in *E. coli* the probability that a substrate participates in k metabolic reactions follows $P(k) \approx k^{-\gamma}$ with γ=2.2 .The power-law connectivity of these network is more resistant to random mutations (Barabasi, 2000).

The connectivity of transcrptional circuits was examined in a quarter of the entire E. coli transcriptional network, the latter being expected to encompass approximately 300-350 genes. It has been estimated that from all transcriptional factors 35% are activators, 43% repressors and 22% dual regulators (Perez-Rueda, 2000). Many regulators autoregulate themselves but there is little cross-transcriptional regulation. Negative feedback is encountered in 40%, positive feedback in 6.5% and dual in 6.5 % of transcription regulators (Thieffry, 1998).

Stability and robustness in cellular networks

Networks display dynamic properties which determine how the temporal changes and steady-state functioning of a system are affected by changing the properties of networks components and topology. Of major importance is the clarification of stability and robustness in cellular networks.

Robustness in control theory refers to the ability of a system to continue functioning in the face of substantial changes to its components (Savagau, 1971). Robustness is qualitatively different from stability (Little, 1999). The latter term refers to the ability of a system behavior to withstand chance fluctuations in the levels of the components in the absence of genetic changes to these components. Such fluctuations might be caused by environmental perturbations, or by the internal stochastic noise of biochemical processes. Robustness, in contrast, refers to the effects of genetic changes in the components.

Robustness in biological systems means that while changing the properties of its components (by mutations, overexpression, induction) the proper functioning of the system is preserved. Robustness can be based on gene *redundancy*: multiple copies of identical or functionally

similar genes ensure the proper functioning, even if mutations impair some of these genes (Thomas, 1993). A less obvious possibility is that a robust behavior is a property of a network of genes lacking functional similarity (Wagner, 1996). In this case modeling can shed light on the basis of robustness. How often can robustness be attributed to the network architecture? In yeast, there are indications that robustness cannot result from the presence of functionally similar 'back-up' genes indicating that robustness is based on other factors (Wagner, 2000).

Conditions for robustness. While there are no general rules how to construct a robust network, different general network topologies can show different degrees of robustness. Inhomogeneous networks are more resistant to mutations than homogenous ones. Interestingly, on large scale, metabolic networks are inhomogeneous: the majority of components have only one or two interactions but a few components have a large number of interactions (Albert, 2000). This property is not shared by other types of networks, such as regular networks. In a model inhomogeneous gene network the majority of components have few interactions, and mutations affect by law of probability these components present in high amount. The impairment of these genes has no impact on the overall network topology and thereby the functionality of the displayed biological process.

A detailed analysis of robustness was performed on bacterial chemotaxis (Barkai, 1997; Alon, 1999). The chemotaxis is characterized by several properties. Some properties, such as the steady-state behavior and adaptation time, show strong variations in response to varying protein concentrations. In contrast, the precision of adaptation does not vary with the protein concentrations.

Robust and sensitive networks in living organisms. Some biological processes function well in spite of perturbation by genetic or environmental factors. Others do not. This difference can be appreciated by considering the effects of mutations in genes controlling embryonic development. Mutations that remove one of the two copies of a gene often cause observable defects in the animal. A well-known example is the small eye mutant of the mouse, in which removing one copy of the pax6 gene causes the eyes to be smaller than normal; whereas removing both copies leads to complete failure of eye formation. The process controlled by pax6 is sensitive to a 2 fold change the amount of gene product (Hill, 1991; Schedl, 1996; Ton, 1991). Other developmental processes are much less sensitive to the levels of gene products. Segmentation of the Drosophila embryo is not affected by 2 fold changes in the expression of many segment polarity genes. Embryos carrying mutations in one or more of these genes can develop normally. A modeling of Drosophila segment formation showed that robustness cannot be gradually depleted while maintaining functional segmentation. If this was the case, removal of some interactions would weaken the robustness of the model,

without precluding a successful outcome. In a less robust system, removing certain gene products in one copy might cause developmental defects (i.e. become haploinsufficient). However, in the cases examined by von Dassow et al, segmentation does not occur at all if the robust core topology is not provided. It raises the possibility that in some genetic networks robustness and functionality are not separable. The biological process is then either robust or completely non-functional.

In many aspects of development, it is well known the robustness of the system can be compromised, without complete failure. Developmental genetics labs take advantage of weakened robustness to perform "sensitized" genetic screens. The genetic makeup of the animal is compromised in a way that makes the system sensitive to alteration in the doses of other genes acting the same biological process. This has been used to great effect in identifying genes involved in the RAS pathway in Drosophila (Rubin, 1997). This allows large numbers of animals to be scored for genetic interactions that would be too subtle to be detected in an animal where the process was fully functional (i.e. as robust as possible).

Feedback control in cellular networks

Feedback loops are ubiquitous regulatory modules of cellular networks. Various effects have been ascribed them (Freeman).

(1) Temporal control of signaling. Cytokins induce the expression of inhibitors (SOCS1) through the JAK/STAT signaling pathway, which in turn decrease signaling.

(2) Spatial control. Both positive and negative feedback are exploited to limit the effect of differentiation factors to appropriate regions of a developing embryo (for instance, hedgehog signaling in Drosophila).

(3) Stabilization of protein expression levels. Keeping the expression level of growth-factors is particularly important to prevent uncontrolled cellular growth. Upon TGF-βsignaling specific Smad proteins are phosphorylated which, in complex with Smad4, activate transcription of inhibitory Smad 6, 7. Inhibitory smads compete either for binding for TGF-β receptors or to Smad4. TGF-β signaling generally inhibits proliferation and consequently overexpression of inhibitory smads is associated with tumors. The conservation of such a negative feedback loop from flies to humans emphasizes its significance. Transcription of the oncogenic snoN is activated when Smad3 enters the nucleus and allows the transcription of snoN by Smad4. SnoN is a corepressor that blocks Smad4-mediated transcriptional activation. In its oncogenic form SnoN is no longer susceptible to Smad3 derepression, so it cannot escape from the negative feedback loop and TGF-β growth inhibition is lost.

(4) Differentiation. Positive feedback causes initially small changes to become irreversibly large, the classic example being the amplification of small, stochastic differences to select a single cell as a neural precursor in Notch-mediated lateral inhibition (Heitzler, 1991).

Spatial aspects of cellular networks

Since the cell is a highly anisotropic medium, activities of many network components do not change only in time but also in space. Such spatial inhomogeneities exert a strong influence on the overall network behavior. Probably, the best characterized localization effects on network dynamics is described in the cell-cycle (Fig 1). A simplified model of the eukaryotic cell-cycle is based on antagonistic interactions between the mitosis promoting factor (MPF) and the anaphase promoting complex (APC). The MPF, which is a dimer of cyclin B and cdc2 (cdk1), acts also autacatalytically by one or more indirect positive feedback. MPF also activates APC, which in turns degrades MPF by a delayed negative feedback (Novak, 1998). The dynamics of the feedback loops is influenced by the shuttling of the network components between the nucleus and cytoplasm via the nucleocytoplasmic transport. The Wee1 kinase, which negatively regulates Cdc2 / cyclin B through phosphorylation at Tyr-15 of Cdc2, is predominantly nuclear, whereas the Myt1 kinase, which can phosphorylate both Tyr-15 and Thr-14, is associated with the cytoplasmic face of the endoplasmic reticulum. Various isoforms of cdc25 phosphatase may be restricted to the nucleus or cytoplasm. Cdc2/Cyclin B1 complexes associate with microtubules during interphase, but transit precipitously into the nucleus at the G2/M transition. The cytoplasmic steady-state localization is dependent on the nuclear export mediator, CRM1. Mutation of the NES leads to a nuclear accumulation of Cyclin B in Xenopus oocytes. Phosphorylation of serines the region containing the NES would reduce the export rate at the G2/M transition. (Yang, 1998). However, the active import of cyclin B would be mediated by a cofactor since it lacks functional NLS sequences. Cyclin F and patched are thought to affect the localization of cyclin B (Barnes, 2001; Kong, 2000). In *Xenopus* Cdc25 contains both NES and NLS-like sequences conferring a continuous shuttling. Binding of 14-3-3 protein to cdc25 markedly reduces the nuclear import rate of cdc25 although it does not greatly affects its activity in vitro producing a <2-fold reduction in activity. However, even a slight increase in cdc25 enzymatic activity resulting from the G2/M loss of 14-3-3 binding might me sufficient, after concentration in the nucleus, to activate a small amount of nuclear cdc25-cyclin B; this would effectively set in motion a positive feedback loop (Yang, 1999). Imposing changes in the localization properties of the cell-cycle components results in premature onset of mitosis or failure of nuclear envelope breakdown (Takizawa, 2000).

Nuclear transport dynamics

Identification the physical-chemical principles governing nuclear transport is necessary for the understanding how the spatial changes in network components arise and what are their

effects. : Proteins tagged with a nuclear export signal (NES), are exported from the nucleus by binding to the export receptor CRM1 in the presence of RanGTP. Ran is a GTPase, which fuels the transport process (Mattaj, 1998) . Upon translocation through the nuclear pore complex (NPC), the CRM1–cargo-RanGTP ternary complex is dissociated in the cytoplasm due to the hydrolysis of the bound GTP. The nuclear and cytoplasmic localization of RanGEF (guanosine nucleotide exchange factor, RCC1) and RanGAP (GTPase activating prorein), respectively, create an asymmetry in the nucleotide bound state of Ran across the NPC. During nuclear transport, the loading of nuclear Ran by GTP and hydrolysis of the bound GTP to GDP in the cytoplasm, generates an out of equilibrium reaction cycle. In this way, the exportin CRM1 promotes the transport of NES carrying cargos along a decreasing RanGTP concentration gradient.

The diffusion of transport receptors is influenced by the NPC. Many nucleoporins bind receptors in a RanGTP dependent way and a structural asymmetry of the NPC is also revealed. There might be a gradient of binding affinities of nucleoporins to transport receptors along their path through the NPC. Thus, nuclear transport provides an example for a transport process where a nonequilibrium chemical reaction is coupled to diffusion in an anisotropic medium. In such processes, Brownian motion of transport cargoes can be biased by a coupled reaction and conversely, spatial concentration fluctuations can substantially modify the biochemical reactions. By this analogy, the exportin-cargo complex might move across the NPC either by random diffusion or by energy dependent directed motion. The motion of the complex might be restricted by NPC gating. However, the resulting effects are critically dependent on the degree to which the Ran dependent nucleotide exchange and hydrolysis reactions are depart from the equilibrium and how the reaction is coupled to the structural anisotropy of the NPC. Therefore, it is crucial for studying nuclear transport to design experiments for in vivo conditions, in which the natural flux rates of the reactions are preserved.

Thus, nuclear transport could be achieved by a wide range of possible mechanisms, yet the actual mode by which diffusion is coupled to energy conversion, has not been addressed. The usual description of nuclear export includes a unidirectional motion of CRM1-cargo complex in the NPC and upon translocation and GTP hydrolysis the unloaded transport receptor reenters the NPC. However, there is no experimental evidence that a unidirectional motion occurs or whether the GTP hydrolysis provides a checkpoint for restricting the release of non-dissociated export complex and allows the re-entry of only the unloaded transport receptor

My present research focuses on how efficiently is the RanGTP gradient coupled the nuclear protein export and which is the mechanism which provides the unidirectionality of cargo transport.

Mathematical formalism for analysis of gene networks

The behavior of gene networks can be viewed in terms of reaction kinetics. Chemical reactions can be analyzed by kinetic and thermodynamic formalism. Thermodynamics delimits which reactions are possible, however, does not explain the speed of changes of reactants. Kinetic theories, on the other hand, require a more detailed information about parameters. In deterministic kinetic equations, the evolution operator assigns always unique values for the subsequent states. In stochastic equations the evolution operator assigns only probabilities for kinetic variables. The mathematics of stochastic process undergoes a rapid development. Many of the basic theorems and derivations are just several decades old. The excessive inclusion of randomness into the models might lead to equivocal and arbitrary interpretations when no attention is paid to convergence criteria and possibility for experimental verification of different theoretical assumptions and consequences. Here, the summary of approaches focuses on how the different equations relate to the physical reality. Short mention are made for the following stochastic formalisms: master equation, stochastic differential equations and the Fokker-Planck equation. For a more detailed treatment of these formalisms can be found in (Gardiner, 1990; van Kampen, 1984; Erdi, 1992)

Stability analysis in deterministic systems

A system of kinetic differential equations can be defined with vector variables: $d\mathbf{x}/dt=f(\mathbf{x})$ and it has a general solution $\mathbf{x}(t)$. Stability provides information about the resistance of a system to perturbations, i.e. how fast the effect of a perturbation decays or how far a system is departed from its original state by a perturbation. The following definitions of stability are used in the dynamical systems theory:

Attracting: x^* is ***attracting*** if there is a $\delta>0$ such that $\lim_{t\to\infty}x(t)=x^*$ whenever $||x(0)- x^*||<\delta$, where x^* is the stateady-state value of $x(t)$.

x^* is ***Liapunov stable*** if for each $\varepsilon>0$, there is a $\delta>0$ such that $||x(t)- x^*||<\varepsilon$ whenever $t\geq 0$ and $||x(0)- x^*||<\delta$

x^* is ***asymptomatically stable*** if it is both attracting and Liapunov stable.

When a fixed point is Liapunov stable but not attracting, it is called ***neutrally stable***. Nearby trajectories are neither attracted nor repelled from a neutrally stable point.

The classical stability analysis of time-independent linearized systems starts from the characteristic equation of the Jacobian matrix. It must be investigated whether this polynomial has only roots with negative real parts in order to guarantee the local stability of the steady-state. The *Routh's* and *Hurwitz'* stability criteria examine the roots of the characteristic equation without actually solving this equation. The Hurwitz' stability criterion is preferable for algebraic stability analysis. The algebraic analysis is preferable to a numerical analysis.

The second method of Liapunov. The Liapunov function is a scalar function V(x) which is the integral of f(**x**) (∫f(**x**)d**x**). In the second method of Liapunov, the derivative of the Liapunov function, the *Eulerian derivative*

$$\dot{V}(x) = \frac{\partial V}{\partial x_1}\dot{x}_1 + \frac{\partial V}{\partial x_2}\dot{x}_2 + \cdots + \frac{\partial V}{\partial x_n}\dot{x}_n$$

which can also be written as the dot product of the two vectors grad V(x) and kinetics x' of the system

$$\dot{V}(x) = grad\ V(x) \cdot \dot{x}$$

By making use of Liapunov functions, some information about the asymptotically stable systems can be obtained without solving the system of differential equations. One can define the *Liapunov exponent*

$$\eta = -\frac{\dot{V}(x,t)}{V(x,t)}$$

Integrating the above expression yields

$$V(x,t) = V(x_0,t_0)\exp\left(-\int_{t_0}^{t} \eta\, dt\right)$$

which shows that the initial value of V(x,t) shrinks to zero as time elapses. Note that η can have different values at different points in the state space. If one chooses the minimal value of η as

$$\eta_{\min} = \min\left[-\frac{\dot{V}(x,t)}{V(x,t)}\right] \quad \text{then} \quad \dot{V}(x,t) \le -\eta_{\min} V(x,t)$$

then

$$V(x,t) \le V(x_0,t_0)\exp(-\eta_{\min}(t-t_0))$$

This expression gives an estimate of the minimal rate by which the steady-state is approached. There are several possible V(x,t) functions for a given asymptotically stable steady-state which results in different values of η_{min}.

Master equation and its approximations

The master equation in chemical kinetics takes into account the fluctuations due to the low number of reactant molecules in small volumes or dilute reactions (internal noise).

The master equation relates the probability of finding a system in the microscopic state *y*, at a time *t* P(y,t) to probability changes owing changes into and off the state:

$$\frac{\partial}{\partial t}P(y,t) = T_{in} - T_{out} = \sum_{y'}[P(y',t)w(y' \to y) - P(y,t)w(y \to y')]$$

where w(y' $\mapsto$ y) may be regarded as the probability, during an elementary time interval, that a system changes from state y' to state y.

The step operator is defined by its effect on an arbitrary function

$$E^{+1}f(n) = f(n+1)$$

$$E^{-1}f(n) = f(n-1)$$

An arbitrary gene network can be characterized with the production (A) and degradation (D) rates of its components. For example, A shows how the rate of creation of species i is influenced by the number of molecules of species j. With the step operator, and the matrices A and D, the master equation can be written in the following way:

$$\dot{p}_{q_i} = (E_i^{-1} - 1)(\sum_j A_{ij}q_j)p_{q_i} + (E_i^{+1} - 1)(\sum_j D_{ij}q_j)p_{q_i}$$

This equation can be solved the moment generating function

$$F(z_j,t) = \sum_{q_v=1,\ldots,\infty}\left(\prod_{v=1,\ldots,n} z_v^q\right)f_q$$

Since the degradation term (D) is diagonal (i.e. the degradation rate of a component is dependent only on its own concentration), the moment generating function can be transformed in the following way

$$\frac{\partial F}{\partial t} = \sum_i (1 - z_i)\left(D_i F_i - \sum_j A_{ij} z_j \frac{\partial F}{\partial z_j}\right)$$

The following definitions are given for the vector $J_j=F_j$ and the matrix $K_{ij}=F_{ij}$ (where subscripts on F denote differentiation). After differentiating to the second moments gives:

$$(A - D)J = 0$$

$$[(A - D)K + L] = -[(A - D)K + L]^T$$

where $L_{ij}=A_{ij}J_j$. These linear equations can be solved for means (J) and variances (K).

Simulation of chemical reactions (according to Gillespie)

The difficulties associated with solving the master equation analytically led to the development of numerical approximations of the master equation for chemical kinetics. The most widely used algorithm is developed by (Gillespie, 1972). The fundamental hypothesis of the stochastic formulation of chemical kinetics is that the reaction parameter c_μ which characterizes reaction R_μ ($\mu = 1, 2, \ldots, N$) can be defined as follows.

$c_\mu\, \delta t \equiv$ average probability, that a particular combination of R_μ reactant molecules will react accordingly in the next time interval δt.

The reaction parameter is proportional with the number of molecular collision events and is affected by activation energy of the reaction:

$$c_\mu = V^{-1}\pi d_{12}^2\left(\frac{8kT}{\pi m_{12}}\right)^{\frac{1}{2}} e^{-\frac{u_\mu^*}{kT}}$$

With the following parameters: V: reaction volume, d_{12}: collisional distance, m_{12} reduced mass of reactants, u^*: activation energy.

The key element of this description is the requirement that the reactant molecules always be randomly distributed uniformly throughout in V; that is what set the stage for the introduction of the collision probability $\delta V_{coll}/V$. It has to be critically examined whether this condition is fulfilled in living cells. In the case of an ideal gas, it suffices to require that the non-reactive (elastic) molecular encounters, which serve to randomize and uniformize the positions of the molecules, occur much more frequently than the reactive (inelastic) molecular encounters, which change the population levels species.

The condition that nonreactive molecular collisions occur much more frequently than reactive molecular collisions is a convenient criterion for applicability of stochastic formulation of chemical kinetics for non-anisotropic systems and non-diffusion controlled reactions.

Then, the probability that a reaction will occur in the time interval $(t+\tau, t+\tau+d\tau)$ is given by:

$P(\tau, t)d\tau=P_0(\tau)h_\mu c_\mu d\tau$.

$P_0(\tau)$ is the propbability that at time t that no reaction will occur in the time interval $(t, t+ d\tau)$.

h_μ is the number of distinct molecular reactant combinations for reaction R_μ ; for example for a unimolecular reaction it is proportional to X_j.

Stochastic differential equations

Stochastic differential equations allow for the inclusion of external perturbations (for example, environmental effects on a reaction).

The inclusion of random effects in differential equations leads to two distinct classes of equations.

(1) When an ordinary differential equation (ODE) has random coefficients or is forced by a fairly regular stochastic process, the random differential equations arise and are solved by sample path by sample path as ODEs. E.g.

$$\dot{x} = \frac{dx}{dt} = a(\omega)x + b(t,\omega)$$

where the forcing process b is continuous in t for each ω. For an initial value $x_0(\omega)$ at t=0, the solution is given by

$$x(t,\omega) = e^{a(\omega)t}\left(x_0(\omega) + \int_0^t e^{-a(\omega)t} b(s,\omega)ds\right)$$

(2) When the forcing is an irregular stochastic process such as Gaussian white noise, the equation can be symbolically written as stochastic differentials, but are interpreted as integral equations with Ito or Stratanovich stochastic integrals.

Fokker-Planck equation

The Fokker-Planck equation can be derived from a stochastic differential equation:

(1) $\dot{x} = f(x) + \xi(t)$

f(x) is the right-hand site of a kinetic equation and ξ(t) is a rapidly fluctuating term with <ξ(t)>=0. Such a description can be used for modeling noise-induced transitions in gene circuits (Hasty, 2000). To account for the rapidness of fluctuations the autocorrelation has to be "δ-correlated", i.e., <ξ(t) ξ(t')>=Dδ(t-t'), with D proportional to the strength of perturbation. The equation (1) can be transformed by using the potential of f(x).

(2) $\dot{x} = -\frac{\partial V(x)}{\partial x} + \xi(t)$

Equation (2) can be solved by a Fokker-Planck equation for P(x,t), which is the probability of finding the system in a state with concentration x at time t.

$$\partial_t P(x,t) = -\partial_x(f(x)P(x,t)) + \frac{D}{2}\partial_x^2 P(x,t)$$

The stationary distribution is

$$\frac{d}{dx}\left[f(x)P_s(x)-\frac{D}{2}\frac{P_s(x)}{dx}\right]=0$$

which can be simply written in terms of probability current

$$\frac{dJ(x)}{dx}=0$$

which has the solution J(x)=constant. Suppose the chemical process takes place on an interval (a=0, b=c_{cutoff}). Then we must have J(a)=J(x)=J(b)≡J and if one of the boundaries is reflecting (J(x,t)=0) — and this is the case for a=0 — then both are reflecting, and J=0.

$$f(x)P_s(x)=\frac{D}{2}\frac{dP_s(x)}{dx}$$

which has the *(potential) solution*

$$P_s(x)=Ne^{\frac{-2V(x)}{D}}$$

where *N* is an integration constant, which has to be chosen such that P_{st} is normalized.

$$\int_a^b P_s(x)dx=1$$

and V(x) is the Liapunov potential

$$V(x)=-\int_a^x f(x')dx'$$

The Fokker-Planck equation is particularly useful to describe bistable reactions.

Thermodynamic description

The scalar flow for the chemical reaction (ξ) is:

$$\nu_A A+\nu_B B+...\leftrightarrow\nu_c C+\nu_D D+...$$

$$J_\xi=\frac{d\xi}{dt}=-\frac{1}{\nu_A}\frac{da}{dt}=-\frac{1}{\nu_B}\frac{db}{dt}$$

The chemical potential in an ideal solution is:

$\mu_i=\mu_i^0+RT\ln c_i$

The affinity for a given reaction is then:

$$A=\sum_{k=1}^{m}\nu_k\mu_k-\sum_{l=1}^{n}\nu_l\mu_l=-\Delta G_0+RT(\sum_{k=1}^{m}\nu_k\ln c_k-\sum_{l=1}^{n}\nu_l\ln c_l)$$

The index "k" denotes reactants, the index "l" products.

where $\Delta G_0=\sum_{k=1}^{m}\nu_k\mu_k^0-\sum_{l=1}^{n}\nu_l\mu_l^0=-RT\ln K$

There is a linear phenomenological relation between fluxes and thermodynamic forces of the chemical reaction is

$$J=L\frac{A}{T}$$

Large deviations from linearity between flows and forces are found in states far from equilibrium. These deviations are of particular importance in case of autocatalytic reactions like the process:

$A+B\underset{k_{21}}{\overset{k_{12}}{\Longleftrightarrow}}2A$ where $A=RT(\ln k_{12}ab-\ln k_{21}a^2)\quad J=a(k_{12}b-k_{21}a)$

In this system there are concentration ranges where flows and forces have opposite signs in their dependence on ξ. The negative flow-force characteristic is essential for several typical phenomena like oscillations of concentrations and spontaneous spatial ordering.

Aspects of gene expression in E. coli and S. cerevisiae for gene circuit design

Gene circuit design in *E. coli* takes advantage from the fact that a quite detailed kinetic characterization is available for prokaryotic promoters. However, fundamentally different logic governs gene regulation in prokaryotes and eukaryotes (Struhl, 1999). In order to understand eukaryotic gene networks additional regulatory principles (for example, chromatin effect) have to be taken into account. This is of paramount importance for design of synthetic eukaryotic gene regulatory networks. For instance, a transcriptional switch can originate both from chromatin effect or a bistable autocatalytic system (see Results and Discussion). The experimental detection of transcriptional activity relies on measurements of

reporter gene expression. I used enhanced green fluorescent protein (EGFP) (see Becskei, 2000, 2001) or firefly luciferase (see Introduction, Reporter Genes) for this purpose.

Difference between prokaryotic and eukaryotic transcription

The difference originates primarily from the association of DNA with chromatin. Prokaryotic RNA polymerases initiate transcription in vitro on purified DNA templates efficiently, with the rate and level being determined by the promoter sequences. Repressors either occlude RNA polymerase (RNAP) binding or form repressosome structures in which interactions between repressor molecules bound at distinct sites cause DNA loops (Hochschild, 1998; Geanacopoulos et al, 1999). The nucleoid structure is unlikely to play a general inhibitory role in transcription because simple promoters are easily accessible and fully functional in vivo. However, histone-like proteins are required for repression of certain promoters by their presence in repressosome complexes, where they play a specific architectural role (Werner, 1997). Activators stimulate transcription at inherently weak promoters by increasing the association of RNAP with the promoter via cooperative DNA binding, or by stimulating polymerase activity already bound to promoters.

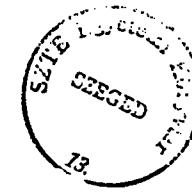
In contrast, eukaryotic chromatin is an inhibitor of protein access to DNA. Nucleosomes virtually prevent the binding of TBP to the TATA element in vitro, and TBP does not associate with yeast core promoters in vivo in the absence, of functional activator. On the other hand, nucleosomes have only a modes inhibitory effect on the ability of a variety of activator proteins to bind their target sites.

Kinetics of transcriptional regulation in *E. coli*

(1) Promoter strength. In vivo promoter strength is not correlated with the promoter recognition measured by the RNAP/promoter association rate. The highest forward rate constants reflect diffusion controlled reactions (Brunner, 1987).

(2) Repressor binding to promoters. Studies using lac repressor showed that there exists no obvious correlation between promoter strength and the repressibility of different operator sequence combinations. Two promoters of almost equal strength *in vivo* differ in their repression by a factor of 10. The efficiency of negative regulation is dependent on low rate of RNAP binding, efficient promoter clearance, and a centrally located operator. (Lanzer, 1988). Various repressors show different association kinetics with operators. The association rate of the *lac* repressor depends on the DNA chain length and on the nonspecific binding constant. Neither of these dependences has been found for the Tet repressor, and , thus sliding does not contribute to any large extent to the recognition of the Tet operator by its repressor.

(3) Positioning of regulatory elements. Study of promoter sequences on a genomic scale revealed that the position of the repressor site shows a strong variation which is in stark



contrast to the relatively fixed position of activator sites. Around 60% of activator sites touch the –40 position. When CRP sites are moved to different proximal positions, the activation is strongest from the *gal* (-40) position, still strong from the *lac* position, and detectable from the *malT* position. CRP was not able to activate from other positions. This positioning might be explained by the fact that promoter recognition elements at –35 and –10. –35 elements are often too far from consensus, indicating weak intinsic promoter binding activity. In a sense, the role of the activator in these cases may be to replace the –35 element and provide a substitute contact point for RNAP (Collado-Vides, 1991).

Yeast transcriptional regulation

The major components of yeast transcription can be divided into three categories: (1) Apparatus for mRNA synthesis. (2) A specific DNA sequence which recruits various components for regulation and mRNA synthesis. (3) Chromatin and chromatin binding activities which modify the accessibility of the promoter to synthetic or regulatory components.

(1) The general components of *mRNA synthetic machinery* include RNA polymerase II and general transcription factors. Cell-free systems supporting basal (unregulated) transcription have been resolved to homogeneity, revealing a requirement for five GTFs, termed TFIIB, -E, -F, -H and TATA-binding protein (TBP). (Kornberg, 1998). The TFIID complex, which contains the TBP and TBP associated factors (TAFs), specifically binds the core promoter region; TBP interacts with high affinity with TATA elements, whereas certain TAFs can interact with some specifity for initiator and downstream elements. (Struhl et al, 1998).

(2) Yeast *promoters* contain three basic kinds of DNA sequence elements.

a) Upstream elements (UAS) are short DNA sequences, typically 10-30 bp in length. Depending on the gene, upstream elements can be located anywhere from 100 to 1500 bp upstream of the initiation site. UAS are analogous to mammalian enhancer sequences in that they function in both orientations and at long distances with respect to initiation site but do not activate transcription when located downstream of the initiation site.

b) TATA elements are typically located anywhere between 40 and 120 bp upstream of the mRNA initiation sites. This is in contrast to most other eukaryotes, in which this distance is fixed (~25-30 bp).

c) The yeast initiator element has varied distance from the TATA element, which might account for why some yeast genes have more initiation sites and TATA elements. The DNA sequence requirements for yeast initiator elements are poorly understood but they are relatively unimportant for determining the rate of transcriptional initiation.

d) Some yeast promoters contain operators for repression and are similar to UAS elements in that they function bidirectionally and at various distances upstream of TATA elements. In general, repression is much more efficient when the operator lies between the upstream and TATA element. One major exception is the mating type silencer that efficiently represses transcription when located at distances as far as 2 kb upstream or downstream from the mRNA initiation sites. (Struhl 1995). Silencing is abolished by mutations in N-termini of histones H3 and H4 (Kayne, 1988). Similar data indicated the effect of chromatin on transcription.

(3) The extent of the *chromatin effect* is exemplified by a study, which shows that loss of nucleosomes in vivo by depletion of histone H4 results in greater than three-fold increase (or three-fold decrease) in expression of some 15 % (or 10 %) of genes (Wyrick, 1999).

Chromatin-modifying activities are classified into (1) chromatin remodeling machines (e.g. SWI/SNF complex) and (2) histone acetyltransferases (HATs) and deacetylases (Gregory, 2001) (see Fig. 2).

Acidic activation domains of activators interact with several different classes of complex involved in the establishment of transcriptional activation. The transactivator Gcn4 interacts with three different classes of such complex: SWI/SNF, SAGA and Mediator. The acidic activation domains of VP16, Gcn4, Swi5, and Hap4 interacts directly with purified SWI/SNF complex in whole-cell extracts. Thus, targeting of SWI/SNF could occur by two non-mutually exclusive pathways. SWI/SNF may be recruited by activators that are previously bound to promoter sequences in chromatin. Alternatively, acidic activators may interact with SWI/SNF in solution and then direct the complex to the promoter region via the sequence specificity of their DNA-binding domains (Neely et al, 1999). Activator recruitment of SWI/SNF leads to localized nucleosome disruption. However, retention of SWI/SNF on the promoter required either the continued binding of the transcription binding of the activator or in the absence of activator histone acetylation as shown with histones purified from sodium butyrate-treated cells. Histone acetylation by either the SAGA or NuA4 HAT complexes increased the retention of SWI/SNF on the promoter (Hassan et al, 2001). The likelihood of a given gene showing dependence upon the SAGA and SWI/SNF complexes is greatly increased if the gene is expressed in mitosis (Krebs et al, 2000).

The yeast Sin3-Rpd3 histone deacetylase complex is required for transcriptional repression by Ume6, a zinc-finger protein that binds URS1 elements. Recently, it has been demonstrated that the Isw2 chromatin remodeling complex is targeted by Ume6 linking the repression function to histone deacetylase (Goldmark, 2000).

The importance of chromatin in transcription is underlined by that the actual binding of Gal4 in the genome is restricted to 10 promoters, which is much less than the number of identical DNA sequences as potential binding sites (Ren et al, 2000).

Reporter genes

Firefly luciferase. Firefly luciferase is widely used as reporter gene since it is sensitive for low transcription rates and ensures a wide dynamic range for detection. The two-step firefly luciferase (LUC) reaction starts with the addition of MgATP or LH_2 (D-luciferin) to the enzyme (Sharon, 1998):

$LH_2 + MgATP + LUC \leftrightarrow LUC\text{-}LH_2\text{-}AMP + MgPP_I$

Step two is the oxidative decarboxylation of luciferin with the production of light on decay of the excited form of oxyluciferin.

$LUC\text{-}LH_2\text{-}AMP + O_2 + OH^- \rightarrow LUC\text{-}OL + CO_2 + AMP + h\nu + H_2O$

The oxyluciferin product, OL, is released slowly from the enzyme-bound complex. This gives the flash kinetic pattern observed with high ATP concentrations (μM), The initial flash of light emission is owing to a "first round" of enzyme activity. This flash decays to a relatively constant light emission, similar to that seen at low ATP concentration (nM), which is thought to be the result of the enzyme slowly turning over by releasing luciferin. This kinetic pattern can be a source of experimental difficulties. When measuring light emission using high ATP concentrations, the delay between starting the reaction and the starting of measurement of light emitted, as well as the length of time that the light emission is measured become critical. In this case, it is essential that the reaction initiated while the sample is within the counting chamber of the luminometer, that the initiating reagent be rapidly and completely mixed with the components already in the reaction cuvet, and that the light emission always measured over the same period of time. These conditions were fulfilled in E coli cell extract measurements. Alternatively, the kinetic pattern can be converted to linear production of light at the high rate of flash by addition of any the following supplements to the basic reaction mixture: 13-20 μM PP_I or 270-500 μM CoA (Stimulates light emission after the flash).

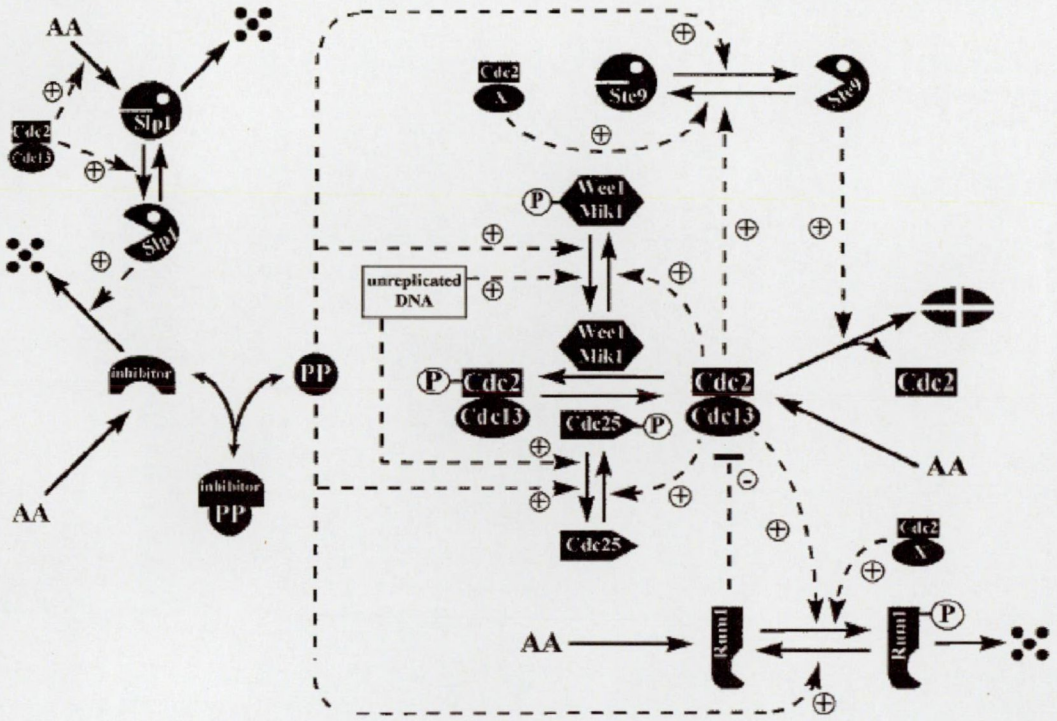


Figure 1. The network of the fission yeast cell cycle. The network topology contains many regulatory elements. Simple activation or inhibition is achieved often by phosphorylation. Autocatalysis is observed between MPF and cdc 25. The fission yeast MPF consists of cdc2-cdc13 complex, in which cdc13 is a cyclin B homologue. Transcription and nuclear transport events are not shown. Reproduced from Sveiczer, 2000.

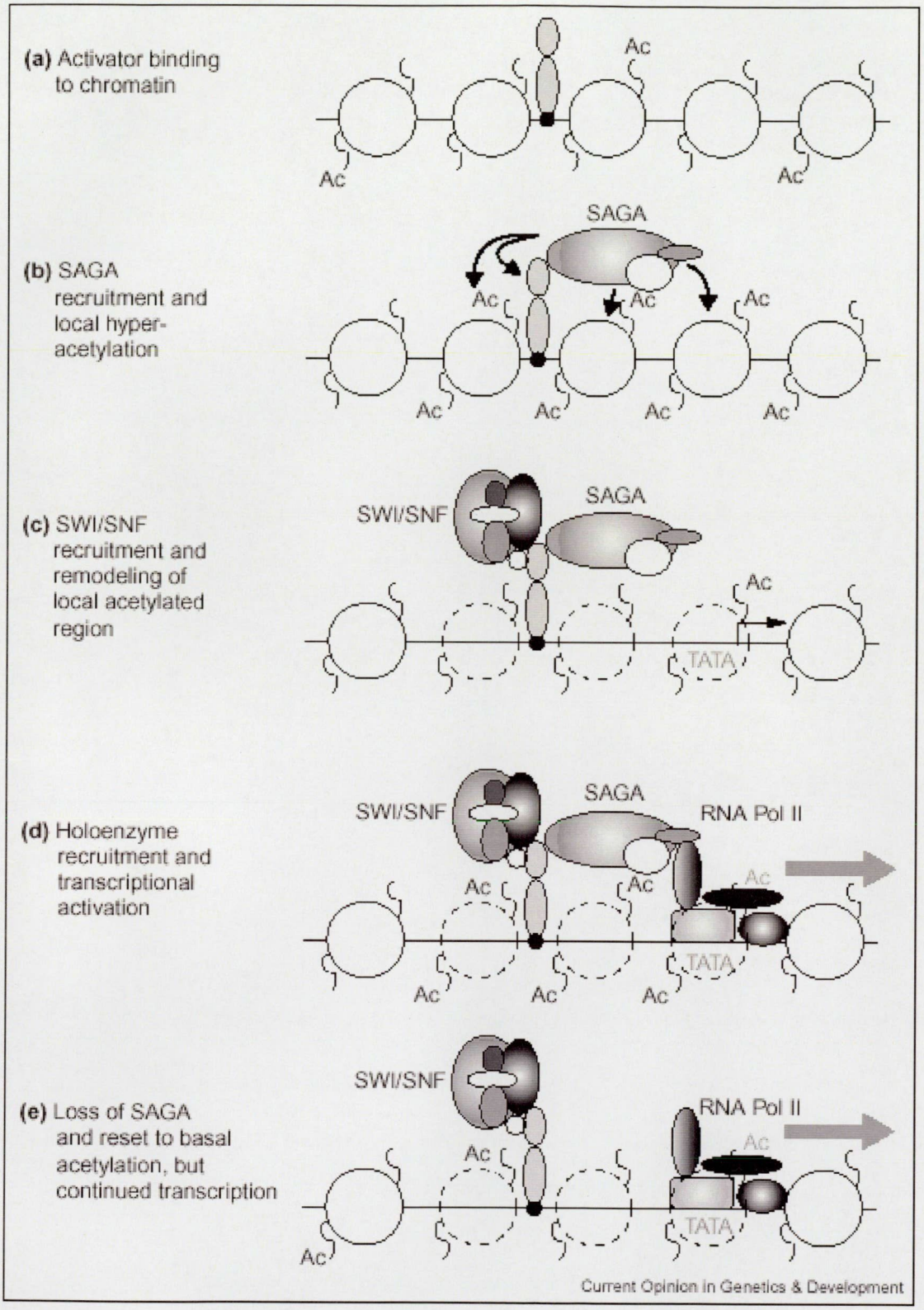


Figure 2. Steps of eukaryotic transcription initiation. Reproduced from (Gregory, 2001).

MATERIALS AND METHODS

Methodology of E. Coli gene network construction

Transformation of chemocompetent *E. Coli* cells

10-12 colonies of DH5α cells were inoculated into 250 ml of Luria Broth (LB) medium in a 2l flask and the culture was let grown at 18°C (200-250 rpm) up to optical density of 0.6 units A_{600}. The culture was incubated on ice for 10 min and centrifuged at 2500 g (4000 rpm) for 10 min at 4°C. The pellet was resuspended in 80 ml of ice cold TB and incubated on ice for 10 min. After spinning down the pellet was resuspended gently in 20 ml of TB. DMSO was added with a gentle swirling to a final concentration of 7% and incubated on ice for 10 minutes. Cells were aliquoted and quick-frozen in liquid nitrogen. This protocol was efficiently used for the following Escherichia coli strains:

XL1-Blue

BL21(DE3)

DH5a

DH5aZ1

HB101 [supE44 ara14 galK2 lacY1 D(gpt-proA)62 rpsL20 (Str) xyl-5 mtl-1 recA13 (mcrC-mrr) HsdS-(r-m-)]

MH1160 MC4100 ompR101

KEG32 MC4100 ompR101 envZ::Tn10

MC4100 Dlac(argF-lac)U169 araD139 rpsL(Str) thiA flbB relA F-

TB (Transformation buffer):

10 mM PIPES	Mix all components except $MnCl_2$ adjust the
55 mM $MnCl_2$	pH with KOH to 6.7.
15 mM $CaCl_2$	Then dissolve $MnCl_2$, filter with 0.45 µm
250 mM KCl	filter. Store at 4°C.

Firefly luciferase activity measurements in *E. coli* cell extracts

1 ml of bacterial culture (OD_{600}=0.3-0.6) was spun for 5 min at 12000 rpm.

The pellet was redissolved and incubated in 30 µl Lysis Buffer (1 mM EDTA, 1 mg/ml Lysozyme) at room temperature (r.t.). Osmotic breakage of cells was achieved by addition of 300 µl dH_2O within 2 min. Then 300 µl of solution A was added. The extract at this stage can be stored at 4°C for several days. For measurements 10-50 µl of extract was mixed with 250 µl of solution B and applied into luminometer cuvettes. 100 µl of 0.25 mM luciferin solution (in dH_20) was injected automatically. Data were collected in integral mode for 10 or 30 sec. Each extract is measured 2x, by deviations above 15% reiterations followed. The optimum temperature for firefly luciferase is 25°C.

For luciferase measurement of living cells, 100-200 µl of growing culture was directly transferred into cuvets, which after an incubation at r.t for 10 min is measured as described above (Knaurs, 1990). The following conditions are observed in usual cases of in vivo measurements: (1) No luciferase activity in culture medium supernatant. (2) Addition of ATP does not influence measured data. (3) Luciferase activity is increased 10 times if the cells are assayed in 100 mM Na-citrate pH 5.0. (4) The activity is increasing in the first 5-10 min and a plateau is reached for several min, followed by a slow decrease. This kinetics is similar to that observed in in vitro measurements with low ATP concentration. The low permeation of luciferin might contribute to this effect. (5) The in vivo measured data can be up 30 to 100 times less than seen in vitro measurements.

Solution A	**Solution B**
100 mM KH_2PO_4 (pH=7.8)	Gly-gly 250 mM
1 mM DTT	$MgSO_4$ 15 mM
	ATP 2.5 mM

Methodology of yeast gene network construction

Yeast transformation

1. Day D-1: In the evening, a fresh (less than two weeks old) culture of the appropriate strain was diluted into 50 ml of YPD. (Usually a 1:1000 dilution around 18-19h00 is appropriate). The culture

was incubated overnight at 30°C by vigorous shaking. 50 ml culture corresponds roughly to 8 transformations.

2. Day D: When OD_{600} reaches 1.0, cells were spun for 5 min at 5 krpm in the Sorvall SS34 rotor. The cells were washed with 50 ml of sterile 10mM TrisCl pH 7.5 or ddH_2O and spun again, as above. Cells were resuspended in 25 ml of sterile LiT (see below) and 0.25 ml of sterile 1M DTT was added and incubated at room temperature for 40 min with gentle shaking.

3. While the cells are incubated, the PEG solution (see below) was prepared as well as the transformation tubes. In each Eppendorf tube 50 ml of sterile LiT, 5.ml of carrier DNA (see below) and 0.1-1 μg of transforming DNA were mixed.

4. After the 40 min incubation, the cells were spun as before and resuspended in 0.75 ml of sterile LiT and 7.5 ml of sterile 1M DTT. 100 μl of competent cells was added to each transformation tube and incubated at room temperature for 10 min.

5. 300 μl of PEG solution was added and the cells were incubated at room temperature for 10 min. (Optional / strain-dependent: 50 μl of DMSO can be added). The incubation at 42°C lasted for 15 min.

6. Cells were spun for 10 seconds and the supernatant was removed. 1ml of YPD was added and incubated at 30°C (or as required) for 60 min.

7. Cells were spun for 60 seconds and 100 μl of 10 mM Tris-HCl pH 7.5 was added.

50 μl was plated in the appropriate selective plate.

Incubation at 30°C (or as required) for 2-3 days.

LiT:

10 mM Tris-Cl pH 7.5 2.5 ml of 2M stock 100 mM LiOAc 5.1 g (MW 102.)	Sterilize by autoclaving or filtration.

Carrier DNA:

Sonicated salmon sperm DNA (or equivalent) at 10 mg/ml.	The DNA is denatured for 10 minutes at 100°C before transformation to ensure the sterility of the carrier.

PEG (polyethylene glycol) solution:

PEG 3350/4000 2 g	LiT 2 ml

This solution is prepared always fresh and sterilized by filtration.

Yeast plasmid and genomic DNA miniprep

1. Cells were grown 2-3 days to saturation in 2ml of appropriate medium (Plasmid recovery is usually better if cells were grown in minimal selective media).
2. The culture was spun (1.5 to 1.8 ml) in an Eppendorf tube for 5 min at room temperature. The pellet was washed with 1 ml water (vortex and spin again).
3. 150 µl of the following mixture was added:
(1.2 M SCE 5 ml, β-mercaptoethanol 50 µl, lyticase 200 U)
4. The cells were resuspended by vigorous vortexing and incubated at 30°C for 30 min. If and only if, the DNA is not to be used for transformation,1 µl of diethylpyrocarbonate was added in each tube. The tubes were mixed by inversion and incubate at 65°C for 30 min.
5.100 µl of 5M K-acetate was added, mixed by inversion, and incubated on
ice for 60 minutes.
6. Tubes were spun for 10 minutes in cold room. The liquid phase was transferred to a new tube, 700 µl of isopropanol was added, mix by inversion and leave at room temperature for 10 minutes.
7. After spinning for 10 seconds at room temperature, to each tube 200 ml of TE +1ml of RNase (Dnase free, 10 mg/ml) was added and mixed well (with a pipette tip) to resuspend the pellet.
8. After incubation at 37°C for 30 min, 20 µl of 3M Na-acetate and 450 ml of ethanol was added and mixed and then incubated at -20°C for 60 minutes.
9. The tubes were spun for 15 minutes, washed with 1 ml 70% ethanol and dried for 5 minutes in Speedvac. DNA was dissolved in 40-100 ml of TE at 4°C overnight. Before use, tubes were spun for 5 minutes to pellet non-soluble particle. Usually, 5 µl was used for a digest (Southern-blot).

Lyticase:
Dissolve the lyophylized powder in:
10 mM Na-succinate pH 5.0 (or Na-citrate)
250 mM NaCl 0.002% NaN3

Store at 4°C up to one year. I used 200 U/ml solution.

SCE:
sorbitol 1.2 M 218 g
Na-citrate 0.1 M 29.4 g
EDTA 75 mM 22.3 g
Water to 1 l

Adjust pH to 7.0 with 5 M NaOH, autoclave.

Southern blot of genomic DNA and hybridization

DNA fragments were separated in a 0.4 % agarose gel.
Depurination: The gel was treated with 0.25 M HCl for 30 min.
Denaturation: After rinsing with water 10 gel volumes of 0.4 M NaOH was poured into the dish and shaken slowly on a platform shaker for 20 min.
With a positively charged nylon membrane, the transferred DNA becomes covalently linked to the membrane if an alkaline transfer buffer is used. Transfer to a charged membrane (Hybond N+) was performed using 0.4 M NaOH as a transfer solution. UV-crosslinking is optional in this case.
The membrane was placed in a hybridization tube and 1 ml of prewarmed (68°C) APS was added per 10 cm^2 of membrane and incubated at 68 C for 15 minutes.
Then the denatured DNA probe was added and incubated with rotation overnight. After incubation the APH solution was disposed of and an equal volume of 2xSSC/0.1% SDS was added. After incubation with rotation (15 min) at r.t. the solution was replaced by an equal volume of 2xSSC/0.1% SDS and incubated as previously. If desired further washed can be carried out at 42 C (moderate stringency) and subsequently at 68°C (high stringency).
The final wash solution was poured off. The membrane was blotted to remove excess liquid and wrapped in plastic wrap. The signal was visualized by autoradiography.

Aqueous prehybridization solution (APS) 68°C (per 10 ml)

5x SSC
10% dextrane sulphate (or 8% PEG 6000)
0.5% SDS
0.5% milk powder
salmon testes DNA
(For hybridization 9.5 mg/ml)

2.5 ml 20xSSC
2.0 ml 50%
0.5 ml 10%
50 mg and up to 10 ml H_2O
0.1 ml, just before use, denature at 100°C for
5 min and chill on ice.

20x SSC

Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of H_2O. Adjust the pH to 7.0 with a few drops of a 10 N solution. Adjust the volume to 1l with H_2O. Sterilize by autoclaving.

Methodology of nuclear transport in Xenopus oocytes

Analysis of injected oocytes

For analysis of injected proteins were prepared tubes with 250 µl TNE buffer for cytoplasmic fraction and tubes with 250 µl EtOH for nuclei. 2 oocytes were transferred to J-buffer and dissected quickly. 6 oocytes were dissected per tube. Cytoplasm was homogenized in TNE by crushing with pipet tip and spun for 15 minutes at 4°C. 200 µl supernatant was pipetted off and precipitated with 1 ml acetone for 30 minutes to overnight at –80 °C. Acetone precipitates and nuclei (in EtOH) were spun for 15 minutes at 4°C. Supernatant was aspirated with gel loader tips attached to vacuum tubes. Pellets were air-dried and resuspended in 7 µl sample buffer per oocyte and vortexed for 20 min. 14 µl sample was loaded per lane for gel electrophoresis.

Biotinylation of proteins via amino groups

Materials:

GST-NES protein (≥1 mg/ml: 30 µM) in prelabel-buffer (100 mM NaCl, Tris 20 mM pH 7.5, 2 mM $MgCl_2$, 8.7 % glycerol)

N-hydroxysuccinimido-biotin (NHS-B) dissolved in DMSO (stock solution 146 mMM Na-bicarbonate (pH 8.3 – 9.0) freshly prepared.

1. 450 µl of protein solution is supplemented with 50 µl of Na-bicarbonate to yield a pH ~8.3
2. Add NHS-B to 1:50 molar ratio (5 µl stock solution)
3. Incubate the reaction for 1 hour at r.t. with continuous stirring
4. Add 50 µl of 160 mM ethanolamin (freshly prepared) and incubate for 30 min.
5. The residual label was removed by dialysis or repeated concentration-dilution with Nanosep 10 K microconcentrators with HMK buffer.

Labeling of recombinant proteins with radioactive thiophosphate

GST protein containing an heart muscle kinase (catalytic subunit) (HMK) site was labeled by incubation of 18 µM of protein with 25 U of HMK (Sigma) and 50 µCi (5 µl) of [γ-^{35}S]-ATP (Pharmacia) in 50 µl of HMK buffer (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, 12 mM $MgCl_2$) for 30 minutes at room temperature.

Nucleotide exchange of RanGTPase

Unloaded Ran is dialyzed against a solution containing 50 mM Na_3-citrate, 20 mM potassium-phosphate (pH=6.8), 0.5 mM $MgCl_2$ and 8.7% glycerol (RGB buffer). For nucleotide loading EDTA was added for 5 mM and GDP or GTP for 1 mM final concentration. The exchange reaction was left to proceed at r.t. for 30 minutes. Then $MgCl_2$ was added for 20 mM final concentration.

Pull-down assays

For measuring affinities of NES sequences to CRM1 , in 100 µl reaction volume the appropriate GST-NES constructs were added to 10 µl of IgG Sepaharose FastFlow to which 160 pmol z-tagged CRM1 was bound. RanGTP or RanGDP was added at 5 µM. The mixture was rotated for 1 h at 4°C. 20 µl of supernatant was removed for assaying the unbound fraction of GST-NES. Thereafter the beads were washed with 100 µl of RGB buffer for 2 minutes. Proteins were eluted with 30 µl of sodium dodecyl sulfate (SDS-PAGE) sample buffer.

RESULTS AND DISCUSSION

Summary. Expression levels of proteins in cells can be described only with a probability distribution in respect to a cell population or in respect to how expression levels change for a given period in a single cell. The shape of the distribution is affected by fluctuations. Negative feedback in genetic circuits can reduce the variance of the distribution, while positive feedback generates a switch, where the transition between two stable states is induced by noise. A detailed description can be found in the attached reprints.

Noise reduction by negative feedback in gene circuits

Noise and fluctuations have been recognized as inherent properties of cellular networks. However, no experiment has addressed how to measure the degree of fluctuations and the resistance of various network topologies to noise.

Various measures are used for the noise strength in a given system. The coefficient of variation is the ratio of standard deviation and the mean. The Fano factor is the ration of variance and the mean. In the experiments, described in (Becskei, 2000), negative feedback reduces the coefficient of variation in simple gene networks.

According to linear stability analysis, the negative feedback system shows ca. 2-fold higher stability when compared to the control system. While linear stability and Langevin analysis account for external noise (see Introduction), the effect of internal noise on the width of distribution is analyzed by the master equation or its approximations (see Introduction). The stabilizing effect of negative feedback is corroborated by a recent analysis of negative feedback in a simple circuit using the master equation (Thattai, 2001), which shows that the intrinsic noise level is considerably decreased by negative feedback. They obtain the following formulas for mean and Fano factor:

$$< p >= \left(\frac{1}{1+\frac{k_p}{d_r}\frac{k_1}{d_p}} \right) \frac{k_r \cdot \frac{k_p}{d_r}}{d_p} \qquad \frac{\delta p^2}{< p >} = \left(\frac{1-\frac{k_1}{d_p}}{1+\frac{k_p}{d_r}\frac{k_1}{d_p}} \right) \cdot \left(\frac{\frac{k_p}{d_r}}{1+\frac{d_p}{d_r}} \right) + 1$$

Here p is the number of repressor molecules, k_r and k_p, are production rates for mRNA and repressor protein, d_r and d_p are the corresponding degradation rates and k_1 is the repression rate. Calculations using the above formulae show that the unregulated gene expression has a Fano factor of 12 while an efficient negative feedback reduces this value to 4 at a repressor binding constant of $K_D \approx 100$ molecules (Thattai, 2001).

Dosage compensation by negative feedback

Negative feedback has been assumed to be involved in mechanisms of dosage compensation in experimental systems. Theories based on binary logical approximations proposed that negative feedback would keep expression at constant level independently of gene dosage (absolute gene dosage compensation) (Thieffry, 1998). To test this hypothesis in these circuits a bicistronic autoregulatory system was constructed. The TetR-EGFP fusion protein and firefly luciferase ORFs were separated by a ribosomal binding site (rbs) and transformed into DH5α.(Fig 3) For control experiment, simple (monocistronic) luciferase expression was analyzed. Other control experiments, using modifications of the bicistronic system could not be evaluated. This is attributed to the toxicity of TetR-EGFP fusion protein at high expression levels, For example, the mutant form of TetR (Y42A) could not be used as the expression from a vector with ColE1 origin of replication resulted in morphological changes of cells (elongated forms) and cell growth slowed down considerably as well. This might be attributed to the toxicity of the mutant form at higher concentrations. Addition of anhydrotetracycline to bicistronic autoregulatory system should also provide data about the behavior of constitutive systems. However, after induction the ColE1 vectors reacted by a transient decrease of expression, which was followed by a recovery (data not shown).

Luciferase activities were determined using cell extracts (see Methods). A comparison of the autoregulatory circuit and the constitutive expression shows that negative feedback reduces the expression level with increasing plasmid copy number but does not result in an absolute dosage compensation. (Fig 3)

Construction of chromosomal gene circuits in *S. cerevisiae*

S. cerevisiae is an organism particularly suited to gene network engineering. The easiness of chromosomal integration at different sites enables the building of transcription units with desired number of copies. Homologous recombination is the favored choice for chromosomal integration. Integrative transformation is greatly increased by linearization of the plasmid in the region of homology. The frequency of multiple transformation increases from ca. 10 % with 0.01 μg to 50% with 1 μg of DNA. (Plessis, 1993).

Integration of pAB237 and pAB247 (see Becskei, 2001) plasmids into LEU2 locus was enhanced by linearization with EcoRV. 1 μg of DNA was transformed to ensure the occurrence of multiple integration (see Methods). For analysis of chromosomal integration, genomic DNA was extracted as described in Methods. Two series of digestions were carried out to provide an accurate number of integrated copies. BamHI sites are not found in the plasmid so digestion with BamHI gives a single band whose molecular weight increases parallel with the integrated copy number. The digested DNA was blotted and hybridized with random probed primers

transcribed from a yEGFP DNA fragment (see Methods). Restriction digestion of genomic DNA with KasI gives two bands (2.0 kb and 10.8 kb) at single-copy integration and an additional 6.7 kb band at multiple integrations. The 6.7 kb band corresponds to the plasmid size and the intensity of this band correlates with the number of integrated copies, whereas the intensity of the other two bands is unchanged. The strains ABY021-ABY028 contain 1, >5, 1, 2, 4, 5, 3, 1 integrated copies, respectively (see Fig 4).

Noise based switch by positive feedback in gene circuits

Autocatalysis is usually viewed as a means to achieve high and stable expression. However, autocatalytic gene expression produces two distinct protein expression levels (low and high) in a cell population (Fig. 5) A genetic switch can be constructed from two mutually inhibiting repressors which is also termed toggle switch (Gardner, 2000). A comparison of the repressor and activator based synthetic genetic switches reveals that they have the same bistability in terms of dynamical systems theory. However, fluctuations in cellular processes produce a noise based switch in the autocatalytic system while no transition are observed between the two stable states in the repressor based switch. Their differential relation to noise cannot be predicted from a mathematical model.

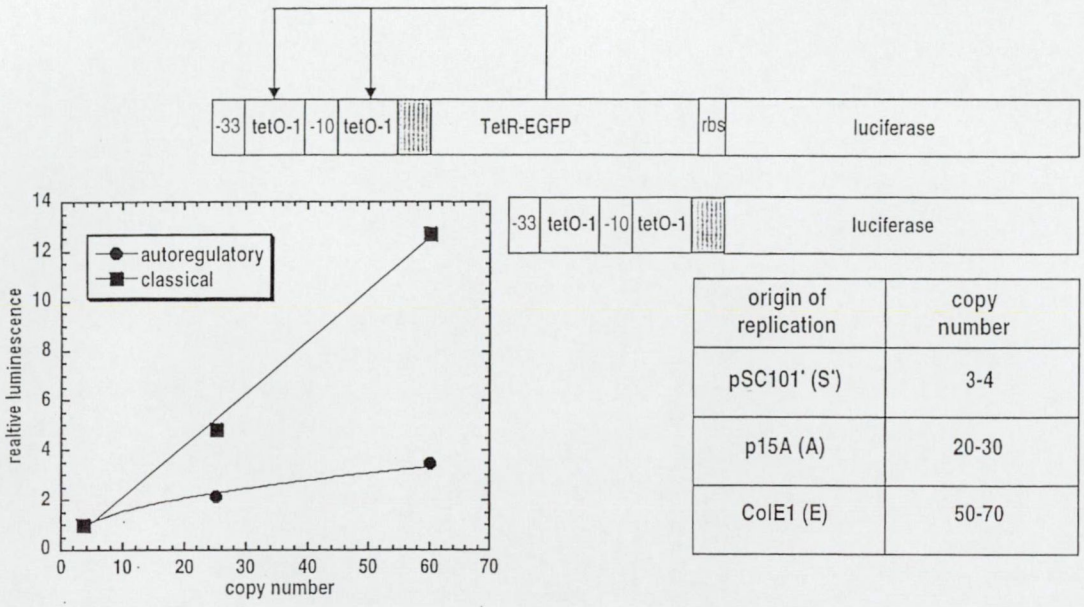
Other mechanisms responsible for on-off type of gene expression

Conversion of a graded response to a switch could be generated by (1) a noise induced transition in a bistable system, and (2) sharpening a sigmoidal response and (3) "chromatin effects".

(1) Noise induced transition in a bistable system. (see previous paragraph)

(2) Sharpening of sigmoid response. Addition of multiple sigmoidal responses results in an increased steepness of the response ("response sharpening"). This phenomenon was observed by Rossi et al (Rossi, 2000) using a modified tetracyclin based transcription system. Their experiments imitate the transcriptional events involved in the establishment of sharp boundaries of gene expression in *Drosophila* at the eve stripe 2 enhancer, which is composed of a cluster of 12 known factor binding sites, six for activators and six for repressors. A GFP-reporter gene was constructed with six TetR binding sequences which was regulated by expressing a repressor and / or an activator. rtTAb binds to the promoter at high doxycycline concentrations as doxycycline induces an activating conformational change in rtTAb. In the absence of doxycycline only the repressor (rTRg) binds to the promoter. At intermediate doxycycline concentration both the activator and repressor bind. The heterodimerization of the DNA binding domains was eliminated by combining the modified version of rTR, the tTRg (Rossi, 1998). A

graded transcriptional response was obtained when either transactivators or transrepressors are present. However, an on/off switch can be seen when these factors compete for the same DNA regulatory element. This conversion can be explained by the addition of two sigmoid curves.
Akin to this mechanism is the signal transduction through a MAP kinase cascade (Huang C-Y, 1996). However, here the sigmoidicity of the response curve occurs due to consecutive reactions, while in the former case simultaneous binding steps produce the same response. The additivity of the responses was also observed in the yeast gene circuits I studied. However, the additivity is generated by consecutive binding steps of transcriptional factors.
(3) "Chromatin-effect" Chromatin-related transcriptional regulation is thought to contribute to on-off type of gene expression. Only 20-30 % of pre-B-cells show expression derived from transgenic mice with integrated early B cell factor (EBF) dependent expression systems embedded into pericentromeric γ-satellite region. Clones were isolated that had 90-100 % of cells either activated or silenced but there was also a range between these two extremes. The fact that a clone expanded to ~2000 cells gave 100% expression indicates that the transcriptionally active state can be inherited. Although relocation to the outside of the heterochromatin complex seems to be a necessary precondition for transcriptional activation, it is not sufficient to give expression. Reducing the dosage of EBF results in a reduced frequency of localization of the transgene to the outside of the heterochromatin complex and lower levels of transcription. (Lundgren, 2000). Similar results were observed in variegation of expression in Drosophila eye development (Ahmad, 2001).



origin of replication	copy number
pSC101* (S*)	3-4
p15A (A)	20-30
ColE1 (E)	50-70

Figure 3. Dosage compensation by negative feedback. The autoregulatory system consists of a bicistronic transcription unit, a TetR-EGFP fusion protein and luciferase separated by a ribosomal binding site, and it is transformed into DH5α strain. The TetR binds to its operator and thereby reduces the promoter activity. In the control system, luciferase is expressed under the regulation of TetR provided by the DH5αZ1 host. Both the autoregulatory and control (classical) system was expressed by three different copy number plasmids (S^*, A, E). Luminescence intensities were measured as described in methods. The intensities of the two very low-copy plasmids were normalized to one. The experiments were repeated four times and different absolute intensities were observed but the behavior of the two systems was the same within the experimental error.

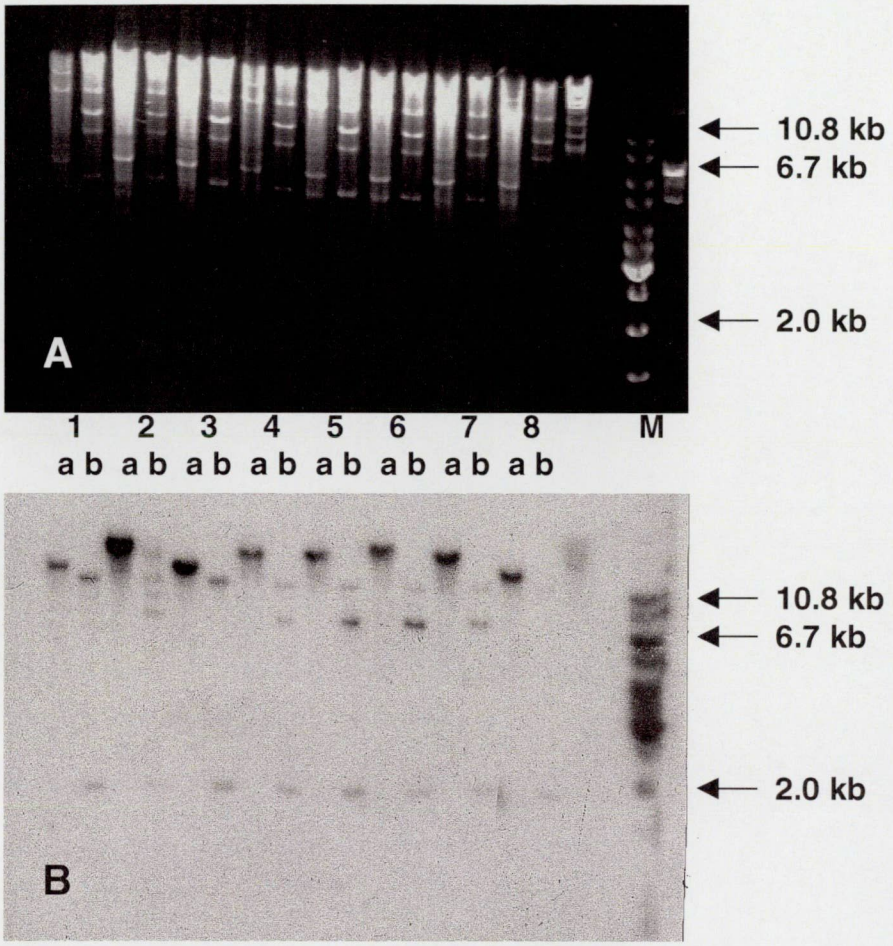


Figure 4. Yeast genomic DNA of strains ABY 021-028 **A.** 0.4 % Agarose gel, stained with etyhidium bromide under UV illumination. **B.** Autoradiography of the Southern blot after random-probe hybridization. DNA of yGFP was used as a template to produce labelled DNA probe by random nucleotide priming. Lanes marked with "a" and "b" denote digestions with BamHI and KasI, respectively. M stands for a 1-10 kb range DNA ladder.

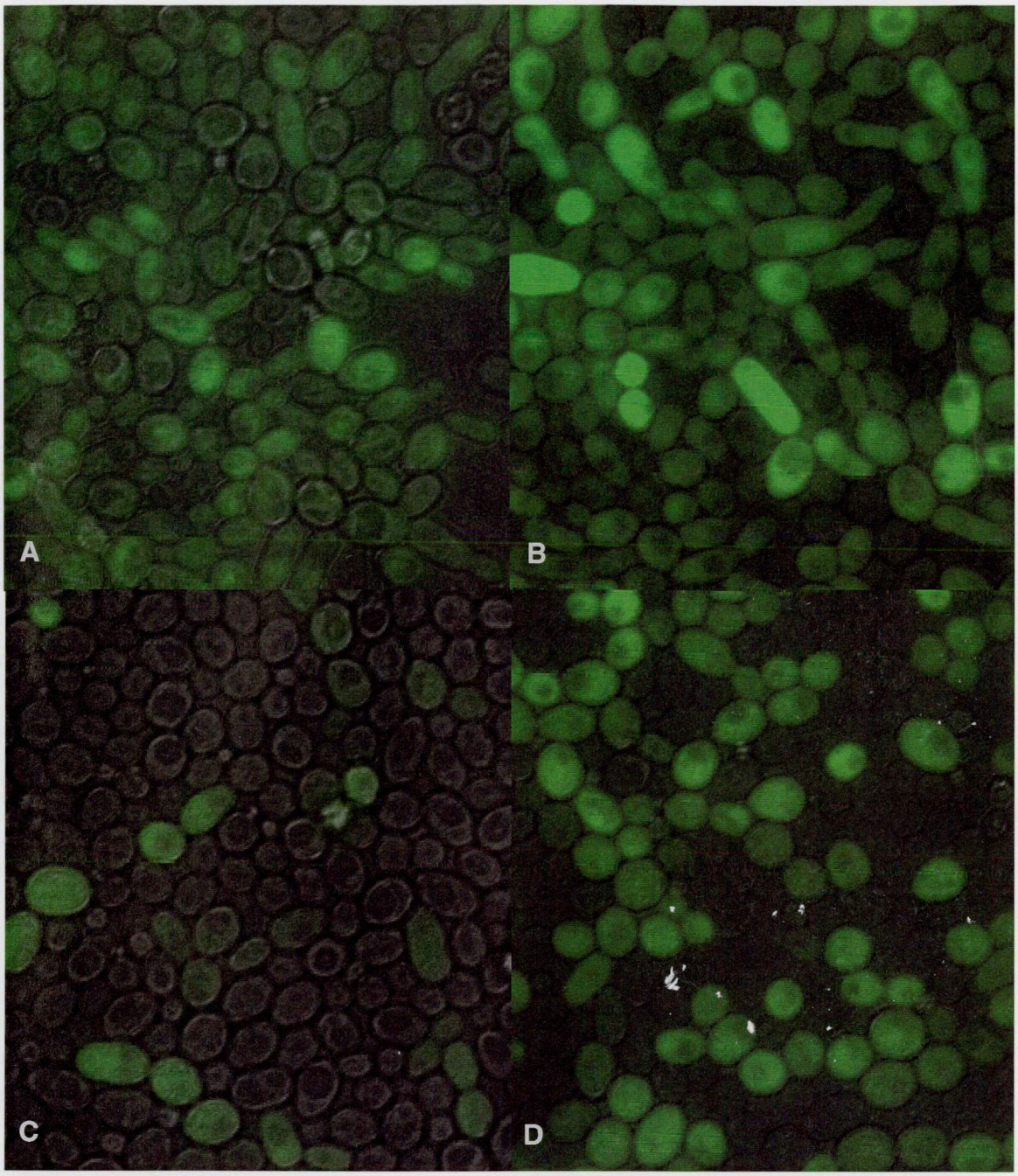


Figure 5. Comparison of the graded response (A, B) to a binary response based on a switch in the autocatalytic gene circuit (C, D). For more details see Fig. 3 in (Becskei, 2001).

OUTLOOK

Living organisms display their defining properties such as adaptability, differentiation and pattern formation, by a combination of random events with deterministic constraints. Despite the non-deterministic effects, organisms have fashioned a stable functioning and development. This robustness lead to the vitalistic concepts in the last century (Kirschner et al, 2000). It is an important task of the present molecular biology to reveal the physical-chemical background of vitalistic properties, which in part originate from an optimal "blending" of random and deterministic processes. The mathematics of stochastic processes tries to develop a comprehensive theory for noise, Brownian motion and superposition of random and deterministic processes. Fluctuations and Brownian motion indeed produce experimentally verified phenomena, which cannot arise based on deterministic principles. For example, a bistable autocatalytic chemical system can amplify input signals at not too low and not too high —optimal— noise level characteristic for stochastic resonance. Brownian motion in anisotropic media can be converted by an irregular energy input into directional motion under certain conditions.

While such research is essentially a basic research in its initial period. Whether applications will ensue is still uncertain. However, a long period elapsed between the description of spin states and antiparticles and their well known applications in modern diagnostic procedures, such as MRI and PET.

Probably, this approach could be useful in the field of "kinetic" diseases. Genetic alterations have dominated the concept of carcinogenesis and little attention was paid to possible changes in the non-structural kinetic changes in mRNA and protein networks. Another possibility is that carcinogenesis might be akin to development, in which no mutations are involved in the entire program of organismic differentiation (Sager, 1997, 1998). It has been recognized that changing the expression levels in many proteins promotes carcinogenesis.. Environmental effects could contribute to the establishment of alternative dynamic states of protein networks with different expression levels and thereby different propensities for cancer development. The combinatorial research could help to observe and interpret such changes. In this case "a network therapy" would overcome genetic abnormalities by inducing differentiation. Induced differentiation by retinoic acid has been proven successful in the treatment of pro-myelocytic leukemia (Fenaux, 1997).

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